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THE ELECTRON TRANSPORT SYSTEM OF *ACETOBACTER SUBOXYDANS* WITH PARTICULAR REFERENCE TO CYTOCHROME *o*

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SUMMARY

1. The nature and distribution of the electron transport system of *Acetobacter suboxydans* (ATCC 621) has been investigated, with particular reference to cytochrome *o*.

2. A highly active membrane-bound electron transport system has been demonstrated, and functional roles suggested for ubiquinone, two *c*-type cytochromes (α peaks at 549 and 553 nm at — 196°), and two *b*-type cytochromes (α peaks at 558 and 564 nm at — 196°).

3. Evidence is presented suggesting that both the *b*-type cytochromes may be terminal oxidases of the cytochrome *o* type, and that cytochrome *o* (558) has an O_2 affinity approx. 10 times greater than cytochrome *o* (565), and a CO affinity only half as great.

INTRODUCTION

Although considerable work has been done on the intermediary metabolism of *Acetobacter suboxydans* (ATCC 621)[1,2], little is known of its electron transport system. Cytochrome difference spectra have been reported, and the presence of cytochromes *b*, *c*, c_1, and *o* suggested[3,4]. Aerobic bacteria, including some other strains of *A. suboxydans*[5,6], exhibit a wide variety of terminal oxidases but these almost always consist of a combination of *a*-type cytochromes, sometimes in association with cytochrome *o*. *A. suboxydans* (ATCC 621), however, is unique in that it is the only bacterium which contains cytochrome *o* in the absence of *a*-type cytochromes[3,4]. This fact, together with its high respiratory rate[7] and lack of some tricarboxylic acid-cycle enzymes[8,9] make it a particularly interesting organism for study: the work undertaken here outlines the general properties of the electron transport system and in particular the properties of cytochrome *o*.

Work on the dehydrogenases of *A. suboxydans*[1,2,10–13] has indicated that many of these are present in different forms, some functioning at different pH, some linked to different coenzymes, and some unlinked: for this reason most results reported here pertain to the NADH oxidase system**.

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** The term 'oxidase system' has been used throughout to refer to oxidative multiple enzyme systems in which only the last member reacts directly with O_2, and the term 'terminal oxidase' to refer to that member of the system which reacts directly with O_2.

A preliminary account of this work[14] and of the effect of glucose[15] on the cytochrome system of *A. suboxydans*, has already been published.

MATERIALS AND METHODS

Maintenance and growth of A. suboxydans

A. suboxydans was maintained and grown as previously described[15], with glycerol as the carbon source.

Preparation of subcellular fractions

Large and small respiratory particles were prepared as described by JONES AND REDFEARN[16].

For some experiments the large and small particles were collected together by omitting the 35000 × *g* centrifugation: these are designated respiratory particles in the text.

All stages of the preparation were carried out at 0–4°.

Measurement of oxidase system activities

Oxidase system activities were obtained by measuring the initial O_2 uptake rate at 30° using a Clark or Rank oxygen electrode. The reaction mixture consisted of 200 μmoles of KH_2PO_4–Na_2HPO_4 buffer (pH 6.5), a suitable amount of particle protein, and substrate, in a final volume of 2.2 ml. 50 μmoles of substrate were used, except for NADH, where 3 μmoles were used.

Spectrophotometry

The concentrations of *c*-type cytochrome and flavoprotein were determined from the $Na_2S_2O_4$-reduced *minus* oxidized difference spectra, using ε_{mM} (551–540 nm) = 19.0 (ref. 17) for *c*-type cytochrome, and ε_{mM} (465–510 nm) = 11.0 (ref. 17) for flavoprotein. Cytochrome *o* was estimated from the $Na_2S_2O_4$-reduced +CO *minus* $Na_2S_2O_4$-reduced difference spectrum using ε_{mM} (417–432 nm, *i.e.* peak–trough) = 170. This was found to give rather more reproducible values than ε_{mM} (417–460 nm, *i.e.* peak–plateau) = 80–90 (ref. 18). As might be expected this gives a ratio of Soret/α peak (417–432 nm/551–568 nm) = 26, approximately twice the ratio found by CHANCE[18] for Soret/α peak (417–470 nm/551–568 nm).

Difference spectra at liquid N_2 temperature were examined in a modified dual-wavelength spectrophotometer by Dr. D. Bendall at the Biochemistry Department, University of Cambridge (Fig. 3). All peaks in this spectrum have been shifted 1–2 nm towards the blue end of the spectrum, compared with room temperature difference spectra.

Dual-wavelength spectrophotometry

The aerobic steady states of the individual cytochrome components of respiratory particles from *A. suboxydans* were measured at 30° using an Aminco–Chance dual-wavelength spectrophotometer, used in the 2–10% transmission range. The reaction mixture contained 250 μmoles KH_2PO_4–Na_2HPO_4 buffer (pH 6.5), 3–6 mg respiratory particle protein in a final volume of 3 ml. The reaction was initiated by the addition of 9 μmoles of NADH.

Wavelength pairs used were 551–540 nm for *c*-type cytochrome, and 565–578 nm for cytochrome *o* (see DISCUSSION).

Photodissociation spectra

Photodissociation spectra were performed by the method of APPLEBY[19], the haemoprotein–CO complexes being excited in the region of the α- and β-absorption bands (525–575 mμ) and dissociation spectra measured in the Soret region.

Identification and aerobic steady-state determination of ubiquinone

To determine the presence of ubiquinone and to identify the ubiquinone homologue, 2 g dry wt. of whole cells of *A. suboxydans* were extracted 3 times with 200 ml chloroform–methanol (2:1, v/v). This lipid extract was fractionated by repeated thin-layer chromatography on silica gel with benzene–chloroform (2:3, v/v) as the solvent. A fraction was obtained with spectral characteristics identical to those of pure ubiquinone. The homologue was tentatively identified by thin layer reversed-phase chromatography with known ubiquinone homologues on paraffin-treated silica gel with acetone–water (9:1, v/v) as the mobile phase.

The method of PUMPHREY AND REDFEARN[20] was used to determine the aerobic steady state of ubiquinone. All experiments were performed at room temperature on *A. suboxydans* respiratory particles.

The reaction mixture consisted of 1 ml of respiratory particles (10–15 mg of protein) in 0.1 M phosphate buffer (pH 6.5): it was vigorously agitated throughout the experiment. The reaction was started by the rapid addition from a spring-loaded syringe of 0.25 ml of a solution containing 10 μmoles of NADH, and was stopped after 1–2 sec, to obtain the aerobic steady state, by the rapid addition of cold (−70°) methanol containing 1 mg/ml pyrogallol. To obtain the anaerobic state the reaction was stopped after 15 min under a stream of N_2; this method gave results which were in good agreement with those in which a Thunberg tube flushed with N_2 was used to obtain an anaerobic state. To determine the percentage of ubiquinone oxidisable by O_2, substrate was omitted and the reaction stopped after 15 min under a stream of O_2.

The quinone was extracted and determined as described by PUMPHREY AND REDFEARN[20].

RESULTS

Activity and components of A. suboxydans respiratory particles

Both pH and the molarity of phosphate buffer exert an effect upon NADH oxidase system activity. The pH optimum is 6.4–6.6 for both large and small particles, and the optimum buffer molarity is 0.1 M. The mean values for NADH oxidase system activities are 2.96 μatoms O_2 per min per mg small-particle protein and 4.12 μatoms O_2 per min per mg large-particle protein. The cytochromes and ubiquinone, together with the oxidase system activities for NADH, glucose, ethanol, and lactate, are all located in the large- and small-particle fraction, rather than in the supernatant (Table I).

The Michaelis constant for the NADH oxidase system in the respiratory particles is 81 μM for NADH. An unusual feature of the Lineweaver–Burk plot[22] for O_2 (Figs. 1 and 2) is the deviation from a straight line at low values of $1/[S]$, *i.e.*

TABLE I

INTRACELLULAR DISTRIBUTION OF RESPIRATORY CARRIERS AND OXIDASE SYSTEM ACTIVITIES IN *A. suboxydans*

Respiratory carrier concentrations are average values, oxidase system activities are those of a typical preparation. Both were determined as described in MATERIALS AND METHODS.

Fraction	*Respiratory carrier (μmoles/g protein)*				*Oxidase system activity (μatoms O_2 per min per mg protein)*				
	Flavoprotein	*c-type cytochrome*	*Cytochrome o*	*Ubiquinone*	*Endogenous*	*NADH*	*Glucose*	*Ethanol*	*Lactate*
Whole cells	0.49	0.26	0.06	0.90	0.02	0.53	2.21	1.59	0.07
French-pressed cells					0.01	1.48	2.15	1.50	0.13
Large particles	1.05	0.80	0 22	4.1	0.01	4.22	5.32	2.89	0.29
Small particles	0.77	0.48	0.11	3.6	0.01	3.32	2.25	1.42	0.18
Supernatant	0.30	0.01	0.01	0.1	0.01	0.05	0.06	0.01	0.01

at high O_2 concentration. This can be interpreted[23] as suggesting the presence of two terminal oxidases. A terminal oxidase with a high affinity for O_2 ($K_m = 2.9$ μM O_2) appears to be active at concentrations of less than 20 μM O_2, and at higher O_2 concentration a terminal oxidase with a lower O_2 affinity is active ($K_m = 33.0$ μM O_2), ignoring the contribution each terminal oxidase must be making to the K_m of the other.

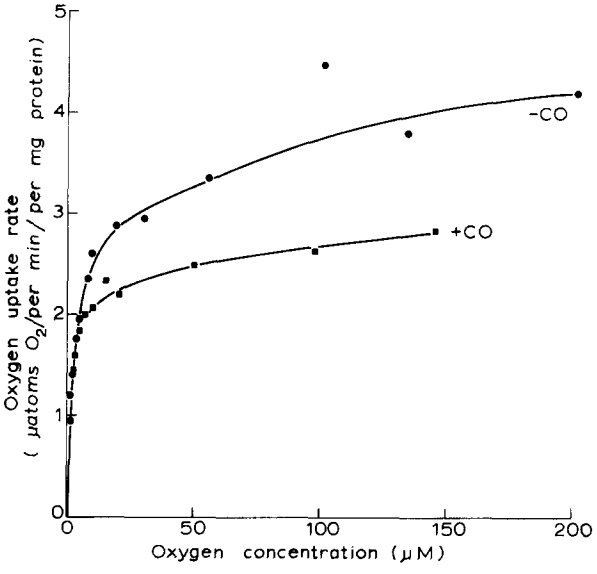

Fig. 1. The effect of O_2 concentration on the NADH oxidase system activity of *A. suboxydans* respiratory particles, in the presence and absence of CO. O_2 uptake was measured as described in MATERIALS AND METHODS: for O_2 levels below 21% the reaction mixture was equilibrated with O_2/A or air/N_2 gas mixtures containing the required proportion of O_2, prior to the addition of particle protein. The particle protein suspension was rendered anaerobic by preincubation with NADH, and was added anaerobically with a micro syringe. Using this technique the O_2 uptake from reaction mixtures containing down to 2 μM O_2 could be accurately measured. The level of particle protein was adjusted so that uptake rates were high enough to be measured over periods of less than 2 min, so that corrections made for the O_2 uptake of the electrode itself, and for the slight diffusion of air into the reaction mixture during the experiment, were very small. For rates in the presence of CO the reaction mixture was equilibrated with a CO/O_2 gas mixture adjusted to give a ratio of 4.5 in solution. A was used to dilute this gas mixture to give successively lower levels of O_2. Frequent controls were run to check against the possibility of electrode poisoning. ●—●, rates in the absence of CO; ■—■, rates in the presence of CO.

Spectrophotometry

In reduced *minus* oxidized difference spectra of respiratory particles from *A. suboxydans* recorded at room temperature an α peak at 551 nm and a shoulder at 565 nm can be distinguished, suggesting that only two cytochromes are present. However, at liquid N_2 temperature (Fig. 3) four components can be seen, absorbing at 549, 553, 558 and 564 nm. The first two of these presumably correspond to cytochromes *c* and c_1 described by SMITH[3]. But since the α peak of reduced cytochrome *o* has been recorded between 555 and 565 nm by different workers (Table II) either or both of the components absorbing at 558 and 564 nm could be cytochrome *o*.

There is no evidence for the presence of any *a*-type cytochrome.

The low-temperature CO difference spectrum (Fig. 3) with a single Soret peak

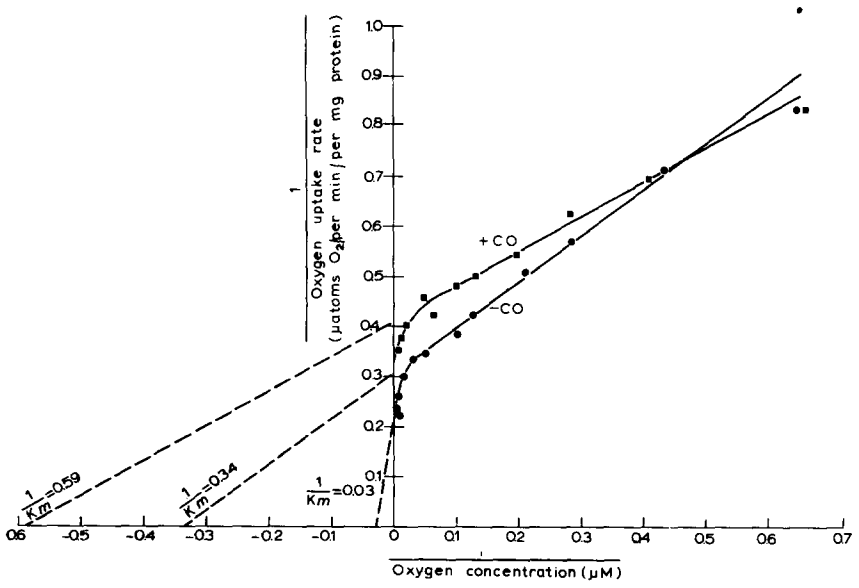

Fig. 2. Lineweaver–Burk plot[22] of the O_2 affinity of the NADH oxidase system of *A. suboxydans* in the presence and absence of CO (from Fig. 1). ●—●, rates in the absence of CO; ■—■, rates in the presence of CO.

TABLE II

ABSORPTION PEAKS OF CYTOCHROME *o*

Reference	*Organism*	*Reduced minus oxidised α peak*	*CO-reduced: minus reduced, peaks and troughs*
SMITH[7,24]	*Staphylococcus albus*	565	567–568, 535–537, 416–418 trough 550
CASTOR AND CHANCE[25]			
CHANCE[26]	*A. suboxydans*	565	567–568, 535–537, 416–418 trough 550
ARIMA AND OKA[27,28]	*Achromobacter*	563	572, 536, 418, trough 554
TANIGUCHI AND KAMEN[29]	*Rhodospirillum rubrum*	564	567, 540, 419–421 trough 560
DANIEL	*A. suboxydans*, (ATCC 621)	557 + 565	568 + 560, 536, 417 trough 551
WEBSTER AND HACKETT[30,31]	*Vitreoscilla*	555 + 565	570, 535, 416, trough 550
BIGGINS AND DIETRICH[32]	*Leucothrix mucor*	558	566, 533, 416, trough 550
BROBERG AND SMITH[33]	*Bacillus megaterium* KM	557	— — 410–415 trough 563–565
IWASAKI[5]	*A. suboxydans*, (IAM 1828)	558	567, 535, 417
TABER AND MORRISON[34]	*Staphylococcus aureus*	555–557	568, 533, 415, trough 556
JACOBS AND CONTI[35]	*Staphylococcus epidermis*	558	573, 537, 417
WHITE AND SMITH[36]	*Hemophilus parainfluenzae*	—	565, 532, 416, trough 547
REVSIN AND BRODIE[37]	*Myobacterium phlei*	—	567, 538, 416, trough 557
REVSIN AND BRODIE[37] and others[7,24,26]	*Escherichia coli*	—	567, 538, 416, trough 558
CHEAH[38]	*Moniezia expansa*	555–557	569–570, 536, 419 trough 556
CHEAH[39]	*Halobacterium cutirubrum*	559	578, 539, 416, trough 560

at 416 nm suggests the presence of cytochrome *o* and confirms the absence of *a*-type cytochrome. The α peak at 566 nm has a shoulder at 558 nm which is not visible in the room temperature CO difference spectrum. It is possible that this shoulder corresponds to a second cytochrome *o*, and that these two α absorption bands in the low-temperature CO difference spectrum may represent the CO complexes of the cytochromes absorbing at 558 and 564 nm in the low-temperature reduced *minus* oxidised difference spectrum.

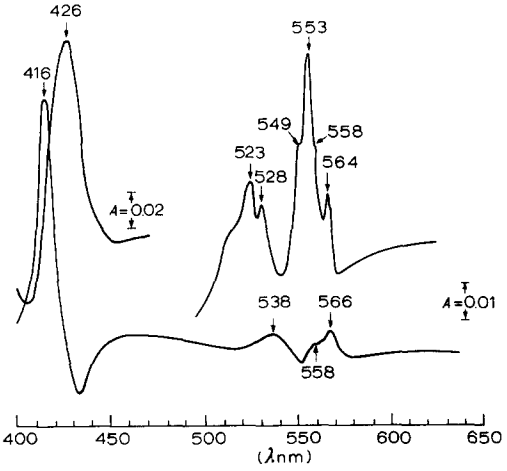

Fig. 3. Low-temperature (77°K) difference spectra of respiratory particles from *A. suboxydans*. Above $Na_2S_2O_4$-reduced *minus* oxidized, below, $Na_2S_2O_4$-reduced + CO *minus* $Na_2S_2O_4$-reduced. A single freeze technique, with dilutions in 0.1 M phosphate buffer (pH 6.5) was used; the light path was 2 mm and the protein concentration was 7.8 mg/ml.

Kinetic experiments with a dual-wavelength spectrophotometer strongly suggest that the cytochrome with the α peak at 564 nm (565 nm in the room temperature difference spectrum) is a terminal oxidase, exhibiting a characteristically very low percent reduction in the aerobic steady state. (Table III). This cytochrome will hereafter be referred to as cytochrome *o* (565). Interference by *c*-type cytochrome prevents the determination of the aerobic steady state of the 558-nm cytochrome.

TABLE III

NADH REDUCTION OF *A. suboxydans* CYTOCHROME SYSTEM AS DETERMINED BY DUAL-WAVELENGTH SPECTROPHOTOMETRY

The determination and experimental conditions were described in MATERIALS AND METHODS. The figures given are mean values, together with the standard error of the mean and the number of determinations. The percentages given refer to that percent of the cytochrome reduced in the aerobic steady state or anaerobic state compared to that quantity of the component reducible by $Na_2S_2O_4$.

c-type cytochrome		*Cytochrome o*	
Aerobic steady state (%)	*Anaerobic state* (%)	*Aerobic steady state* (%)	*Anaerobic state* (%)
47 ± 1.3 (26)	89 ± 1.2 (24)	0.0 ± 1.8 (8)	87 ± 2.4 (8)

Turnover numbers

The respiratory particles of *A. suboxydans* are highly active, and the turnover numbers of cytochromes *c* and *o*, determined by the method of ESTABROOK AND MACKLER[40], reflect this activity. Table IV provides a basis for comparison with other organisms.

TABLE IV

A COMPARISON OF TURNOVER NUMBERS OF *A. suboxydans* CYTOCHROMES WITH THOSE FROM OTHER ORGANISMS

Figures were determined by the method of ESTABROOK AND MACKLER[40], and are expressed as moles of O_2 taken up per mole of respiratory carrier per min × electron equiv. Figures for *A. suboxydans* in parenthesis are maximum values, others are average values.

Source	*Substrate*	*Turnover number*	
		Cytochrome c	*Terminal oxidase*
A. suboxydans	NADH	340(860) *	330(1070)
A. pasteurianum	Ethanol[7]	—	620
Azotobacter vinlandii	NADH[41]	49	147
Yeast	Ethanol	—	65
Heart muscle	NADH[40]	81	72
	Succinate[7]	—	29

* Assuming half of total *c*-type cytochrome to be one particular cytochrome *c*.

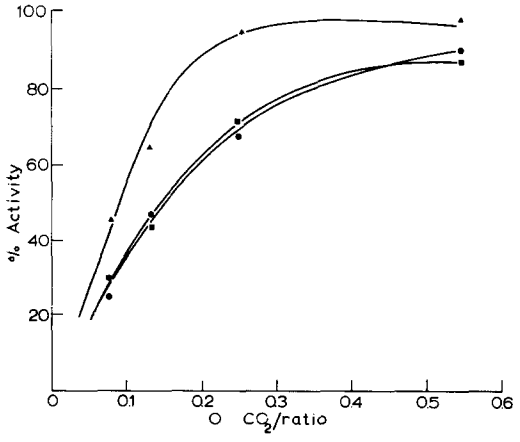

Fig. 4. The CO inhibition of NADH oxidase system activity in the dark (●—●) and in red (■—■) and blue (▲—▲) light. The partial pressure of O_2 was kept constant at 0.20 for O_2/CO ratios (expressed as gas in solution) above 0.35: figures for inhibition at ratios lower than this were necessarily obtained at partial pressures of O_2 lower than 0.2, and may therefore be exaggerated (see Fig. 1). High intensity illumination of the water-cooled clear perspex reaction chamber of the oxygen electrode was provided by a 1000-W spot lamp with a 3-l round-bottomed flask filled with water used as a heat filter and focussing device. A Wratten filter No. 25, transparent to wavelengths above 590 nm, was used to transmit red light only, and the light transmitted through this filter was 20 times the intensity (as measured by the Isco spectroradiometer, Instrumentation Specialities Co., Lincoln, Nebr., U.S.A.) of that transmitted through the blue Wratten filter No. 47B, which was transparent to light in the region 400–470 mμ. The results have been adjusted for the small effect of light on uninhibited preparations. These experiments on CO inhibition were carried out at 0–1°.

Inhibition of NADH oxidation by CO

Fig. 4 shows the inhibition of NADH oxidase system activity by CO and the reversal of this inhibition by light. The results indicate that the CO complex formed with the terminal oxidase is dissociated by blue light. The absence of any red-light effect is consistant with the absence of cytochrome *a*-type terminal oxidases, whose CO complexes all have absorption peaks above 585 nm.

CO affinity and photodissociation of the CO complex of cytochrome o

These properties were investigated to see if the existence of a second terminal oxidase, suggested by the Lineweaver–Burk plot for O_2 (Figs. 1 and 2) and by the low-temperature CO difference spectrum (Fig. 3), could be substantiated.

Fig. 5 shows the Hill plots[19,42] resulting from a CO titration of respiratory particles at four different wavelength pairs. A Hill-plot slope of $n = 1$ would mean the presence of only one type of CO-binding site, without haem–haem interaction; a slope of $n < 1$ could indicate the presence of multiple components with differing CO affinities or of independent and different binding sites on a single component. A slope of $n > 1$ indicates a cooperative effect, with the minimum number of sites involved equal to the slope. Bearing in mind the uncertainties involved in the calculations and corrections used to produce Fig. 5, three of the four slopes do not differ significantly from $n = 1$.

Plots derived from the wavelength pairs 536–551 nm and 568–586 nm indicate the presence of a CO-reactive component with absorption differences at these wavelengths, which is half combined at a CO level of 2 μM. The interpretation of the remaining two Hill plots is less simple. The wavelength pair (560–551 nm) indicates a second component with at least two cooperative CO-reactive sites, since the slope is $n = 1.9$. This component is half combined at a CO level of 3.3 μM, and probably corresponds to the cytochrome whose CO complex absorbs at 558 nm in the low-temperature CO difference spectrum. The fourth wavelength pair (568–551 nm) suggests the presence of another CO-reactive pigment, half combined at a CO level of 6 μM.

However, since the absorbance difference between this last wavelength pair will undoubtedly be affected by the contribution of the 558-nm cytochrome mentioned above, it is possibly more reasonable to assume that this last Hill plot is only the result of contributions from the other two CO-reactive pigments described above.

This CO-affinity experiment was repeated using freshly grown whole bacteria, although the optical properties of these are poorer than those of the respiratory particles. The following results were obtained: for the (536–551 nm) and (568–586 nm) wavelength pairs $n = 1.1$ and the half combination value is 3.8 μM CO; for the wavelength pair (551–560 nm) $n = 2.3$ and the half combination value is 8.1 μM; for the (568–551 nm) wavelength pair $n = 1.2$ and the half combination value is 6.2 μM CO. The figures for the last wavelength pair tend to support the suggestion that these are the result of absorbance components from the other two CO-reactive pigments, rather than of the presence of a third CO-reactive pigment, since the half combination value for CO is intermediate between these other two compounds.

Owing to the poorer optical qualities of whole bacteria, and the lower concentration of cytochrome *o* (Table I), the figures obtained for whole bacteria are probably less accurate than those for respiratory particles. However, in whole cells there is less

chance of any artifact formation or decomposition occurring which might alter CO-binding properties. The similarity of the results obtained from respiratory particles and whole bacteria suggests that such changes have not taken place. But in bacteria which have been stored for over 12 h in ice no slope higher than $n = 1$ can be found, indicating that the component at the wavelength pair 560–551 nm is somewhat labile.

Although the validity of selecting closely adjacent wavelength pairs for this type of treatment may be suspect, since the room temperature CO difference spectrum does not show the CO-reactive pigment absorbing at 558 nm in the low-temperature CO difference spectrum, there still seems little doubt that this pigment is a quite separate entity from cytochrome *o* (565), and it will be referred to hereafter as cytochrome *o* (558).

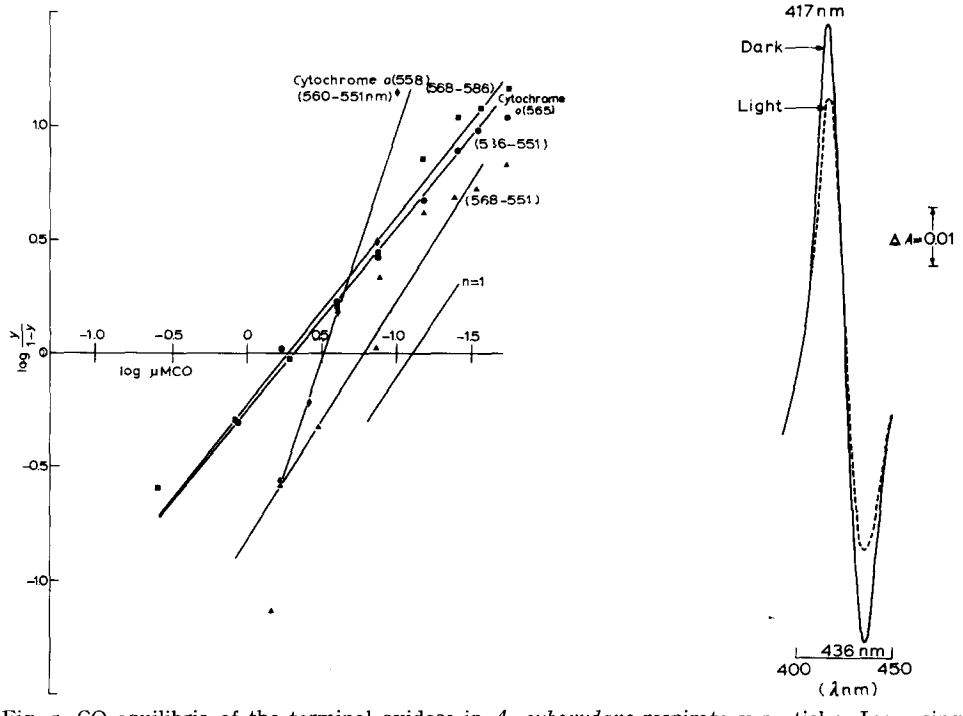

Fig. 5. CO equilibria of the terminal oxidase in *A. suboxydans* respiratory particles. Increasing amounts of CO were added by injection of a 1 mM solution into a filled sample cuvette of 10 mm light path, fitted with a rubber ampoule cap and containing a plastic-coated stirring magnet; both sample and reference cuvettes contained $Na_2S_2O_4$-reduced respiratory particles (8.1 mg protein/ml), and the CO difference spectrum was recorded 2 min after each CO injection. From the Hill plot (*cf.* refs. 19 and 42) $y/(1-y) = KX^n$, where y = fractional combination of each component with CO, K = dissociation constant and X = molarity of uncombined CO: then $\log (y/(1-y)) = \log K + n \log X$. So for a plot of $\log (y/(1-y))$ against $\log X$, the slope will be n, and at half combination with CO $\log (y/(1-y)) = 0$. ●—●, (wavelength pair 536–551 nm) and ■—■ (wavelength pair 568–586 nm), cytochrome *o* (565)–CO complex (see DISCUSSION); ◆—◆ (wavelength pair 560–551 nm), cytochrome *o* (558)–CO complex (see DISCUSSION); ▲—▲, difference plotted at 568–551 nm, probably cytochrome *o* (565)–CO complex + cytochrome *o* (558)–CO complex.

Fig. 6. Photodissociation spectra of *A. suboxydans* respiratory particles. The dark trace (————) is of a $Na_2S_2O_4$-reduced + CO *minus* $Na_2S_2O_4$-reduced spectrum of respiratory particles (3.6 mg protein/ml). The light trace (– – – –) was recorded during subsequent side illumination of the sample cuvette (see MATERIALS AND METHODS) with actinic light (525–575 nm, 3×10^4 ergs·cm^{-2}·sec^{-1} measured at the cuvette surface).

The photodissociation spectrum (Fig. 6) shows only one peak in the Soret region. This could be due either to the fact that one of the CO-reactive pigments is not a cytochrome and hence has no absorption in the Soret region, or that both CO-reactive pigments have absorption peaks at the same wavelength in the Soret region.

The effect of CO on the O_2 affinity of A. suboxydans respiratory particles

Figs. 1 and 2 show the effect of CO on the O_2 affinity of *A. suboxydans* respiratory particles. It is clear from these figures that the oxidase system with the lower O_2 affinity (predominant at higher O_2 levels) is the one most affected by CO. In the presence of CO the apparent $1/K_m$ for O_2 of the high-affinity oxidase system (represented by the straight line portion of Fig. 2, and active at low O_2 levels) is raised from 0.34 to 0.59 μM O_2. This is presumably due to the reduced contribution of the low-affinity oxidase system to the overall O_2 uptake.

Other inhibitors of NADH oxidase system activity

Table V shows the effect of other inhibitors of NADH oxidase system activity. A notable feature is the effectiveness of piericidin A at low concentrations, though the level used is 1000 times higher than that needed to produce complete inhibition in mammalian mitochondria[43]. Studies on the effect of piericidin A on the steady-state levels of the respiratory components of *A. suboxydans*[44] tend to support the suggestion of earlier workers[43,45] that it may act at more than one site in the electron transport system, depending upon the concentration used. At concentrations of 5 nmoles/mg protein (50 % inhibition) it acts early in the chain, prior to flavoprotein and probably at the NADH dehydrogenase level, while at levels of 200 nmoles/mg protein (90–95 % inhibition) piericidin A also acts between *c*-type cytochrome and cytochrome *o* (565). Inhibition by piericidin A is reversible by the addition of lower homologues of ubiquinone but this result is complicated by the fact that these homologues stimulate the uninhibited NADH oxidase system activity by up to 200 % in any case[44,46].

TABLE V

INHIBITION OF THE NADH OXIDASE SYSTEM OF *A. suboxydans* RESPIRATORY PARTICLES

Oxidase system activity was determined as described in MATERIALS AND METHODS. The addition of 0.05 ml of methanol, used as a solvent for some of the inhibitors, had no effect on the assay system.

Inhibitor	*Concentration*		*Inhibition* (%)
	μM	*μmoles/mg protein*	
Piericidin A	1	0.011	35
Piericidin A	10	0.110	85
2-Heptyl-4-hydroxy-quinoline-*N*-oxide	50	0.55	60
Antimycin A	50	0.55	55
KCN	50	0.55	8
KCN	750	8.25	65
CO	O_2/CO = 0.15		50
NaN_3	500	5.5	10
Rotenone	200	2.2	40
Bathophenanthroline	100	1.1	50
Dinitrophenol	300	3.3	30

2-Heptyl-4-hydroxyquinoline-*N*-oxide is also a fairly potent inhibitor of NADH oxidase system activity and dual-wavelength studies again suggest two sites of action, one between flavoprotein and *c*-type cytochrome and another at cytochrome *o* (565)[44].

Concentration and steady-state levels of ubiquinone

Ubiquinone was detected and tentatively identified as Q-10 as described in MATERIALS AND METHODS. This homologue has also been found in other species of Acetobacter[47,48] although it is otherwise unusual in non-photosynthetic bacteria[49].

Tables I and VI show the concentration and steady-state levels of ubiquione, respectively. Due to the high activity of the NADH oxidase system some difficulty was experienced in obtaining consistent values for the aerobic steady state.

TABLE VI

NADH REDUCTION OF *Q*-10 IN *A. suboxydans* RESPIRATORY PARTICLES

Determinations and experimental conditions were as described in MATERIALS AND METHODS. The figures given are mean values, together with the standard error of the mean and the number of determinations. Percentages given are for that percentage of the extractable ubiquinone reduced compared to that quantity oxidisable by chloroauric acid.

Q-10 reduced in presence of O_2 (%) *(substrate omitted)*	*Aerobic steady state* (%)	*Anaerobic state* (%)
$7 \pm 3(3)$	$62 \pm 7.7(6)$	$92 \pm 2.2(6)$

These figures suggest a functional role for ubiquinone in the respiratory chain, at a site prior to *c*-type cytochrome.

DISCUSSION

The work presented has demonstrated the presence of a highly active, membrane-bound electron transport system in *A. suboxydans*. Ubiquinone has been implicated in NADH oxidation, and the low-temperature difference spectrum (Fig. 3) suggests the presence of two *c*-type cytochromes and two *b*-type cytochromes. Dual-wavelength studies (Table III) indicate that neither of the *c*-type cytochromes is likely to be functioning as a terminal oxidase, since although the two *c*-type cytochromes cannot be distinguished by this method, the percentage of total *c*-type cytochrome reduced in the aerobic steady state is even higher than that usually found in bacteria (*cf.* refs. 50 and 51); whereas if one of the *c*-type cytochromes was a terminal oxidase, a lower than usual percentage reduction of the total *c*-type cytochrome might be expected. The very low percentage reduction in the aerobic steady state (at room temperature) of the *b*-type cytochrome absorbing at 564 nm (referred to as cytochrome *o* (565)) in the low-temperature difference spectrum strongly suggests that this cytochrome is a terminal oxidase.

The low-temperature CO difference spectrum suggests that two CO-reactive pigments are present, possibly the two *b*-type cytochromes. The CO inhibition of

oxidase activity and the relief of this inhibition with blue light shows that at least one of these CO-reactive cytochromes is a terminal oxidase. The Lineweaver–Burk plot for O_2 (Fig. 2) can be interpreted as indicating two terminal oxidases, suggesting that both the CO-reactive cytochromes may be terminal oxidases. The Lineweaver–Burk plot for O_2 in the presence of CO appears to show that CO inhibits the terminal oxidase with the low O_2 affinity more than the other. This makes this low O_2 affinity oxidase likely to be a conventional cytochrome type of terminal oxidase rather than a flavoprotein: and since it is known that flavoprotein oxidases tend to have low O_2 affinities[52,53], the other, high O_2 affinity, terminal oxidase is also unlikely to be a flavoprotein. The Hill plots (Fig. 5) show the presence of a CO-reactive pigment, with a single CO-binding site, having absorbance differences at wavelength pairs 536–551 and 568–586 nm: this is probably the cytochrome absorbing at 564 in the low-temperature difference spectrum (cytochrome *o* (565)). A second CO-reactive pigment (referred to as cytochrome *o* (558)) is also apparent, with an absorbance difference at the wavelength pair 560–551 nm, and having a CO affinity about 40–50 % lower than cytochrome *o* (565). Since Figs. 1 and 2 indicate that the terminal oxidase with the high O_2 affinity is inhibited least by CO, *i.e.* has the lower CO affinity, this suggests that cytochrome *o* (558) is the terminal oxidase having the high O_2 affinity.

However, the most striking feature of cytochrome *o* (558) is the Hill plot slope of about 2, suggesting the presence of at least two interacting CO-sensitive sites. This raises the interesting possibility that cytochrome *o* (558) may be a polymeric unit, as is the mitochondrial cytochrome $a + a_3$ complex.

A survey of the literature (Table II) indicates that *o*-type cytochromes, with a few exceptions, can be divided into two groups, one with the main α-absorption peak at 555–558 nm and the other group with the main α-absorption peak at 564–565 nm, in the reduced *minus* oxidised difference spectra. Few, if any, CO affinity studies have been undertaken, and it may be that other cytochromes *o* also exhibit multiple sites of action.

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